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Fate and Toxicity of Aircraft Deicing Fluid Additives Through Anaerobic Digestion

Cyndee L. Gruden, Sarahann M. Dow, Mark T. Hernandez

ABSTRACT: Benzotriazole derivatives are widely used corrosion inhibitors and their fate during wastewater treatment processes is unknown. The purpose of this research was to study the toxic effects and fate of the two commercially significant benzotriazole isomers used in aircraft deicing fluids (4-, and 5-, methylbenzotriazole [MeBT]) during anaerobic digestion. Experiments were executed in microcosms using mesophilic anaerobic biomass co-digesting wastewater sludge and propylene glycol. Sorption of MeBT to digesting solids could be approximated with a Freundlich model, and no anaerobic breakdown of either MeBT isomer was detected. Digesters fed more than 300 mg/L MeBT responded with a significant decrease in methanogenic microbial activity and volatile solids production and a concomitant increase in accumulation of volatile fatty acids. Direct microscopic measurements using fluorescent phylogenetic probes applied to digesting biomass revealed that members of both Archaea and Bacteria domains were sensitive to MeBT. Granular activated carbon (GAC) (volatile solids: GAC = 10%) reduced the apparent toxic effects of MeBT; GAC addition nearly restored the baseline activity of digesters fed MeBT (500 to 1000 mg/L). *Water Environ. Res.*, 73, 72 (2001).

KEYWORDS: toxicity, aircraft deicing fluid, anaerobic, corrosion inhibitors, triazole.

Background

Benzotriazole derivatives are corrosion inhibitors that are universally present in automotive antifreeze, brake fluids, metal-cutting fluids, industrial cooling water systems, and aircraft deicing fluid (ADF). Industry specification requires that corrosion inhibitors be included in ADF (SAE, 1997), and methylbenzotriazole (MeBT) is the compound that is most often used. In addition to its corrosion-inhibiting properties, MeBT is added to ADF to reduce the flammability hazard created from the corrosion reaction that occurs when glycol solutions come into contact with metal components carrying direct current (Downs, 1968). The MeBT used in modern ADF is a mixture of two isomers: 4-methylbenzotriazole (4-MeBT) and 5-methylbenzotriazole (5-MeBT) (Figure 1). The U.S. Environmental Protection Agency (U.S. EPA) recently conducted a study of airport deicing practices to evaluate whether national effluent limitation guidelines and standards are warranted for deicing operations (U.S. EPA, 1999). The first draft of the report provided toxicity data for MeBT, but did not include recommendations for effluent guidelines or permit standards regarding this compound.

Methylbenzotriazole is a weak organic acid (negative logarithm of association constant, pK_a = 8.8), which complexes strongly with some metals. Sorption of MeBT to organic carbon is anticipated because of its relatively hydrophobic nature (log of octanol–water partition coefficient, K_{ow} > 2) (PMC, 1996). The only information available on the potential environmental fate of MeBT is from a U.S. patent, which claims that, in cooling tower waters with sufficient oxygen, 5-MeBT is not stable whereas 4-MeBT is recalcitrant (Rao et al., 1996). Peer-reviewed literature has implicated corrosion inhibitors as the agents imparting significant microbial toxicity to ADFs (Cancilla et al., 1997 and 1998, and Cornell et al., 2000); these compounds were identified as tautomers of benzotriazole and its methylated derivatives. Toxic responses of fish, zooplankton, and soil and marine bacteria have been recently observed with exposure to the concentration ranges of benzotriazoles measured in ground and surface waters at large airports (i.e., > 10 mg/L) (Cancilla et al., 1997 and 1998, and Cornell et al., 2000).

Concerning deicing waste streams and associated runoff, past research has focused on the aerobic treatment of glycols in ADF, but has not included an investigation into the treatability of the other special-purpose chemicals present in modern ADF formulations, collectively known in the industry as *Ad Pack*. These chemicals are present in significant concentrations (> 1% w/w) in domestic-market-grade ADFs and the methylbenzotriazole fraction alone is at or greater than 0.5% by industry specification (SAE, 1997). Previous research has shown that ADF waste can be treated with some modifications to traditional aerobic wastewater treatment processes. However, severe disturbances have been reported when shock loadings of ADF waste have been introduced to some facilities and when low wastewater temperatures have been sustained in the presence of ADF (Krumsick et al., 1994, and Sabeh and Narasiah, 1992). Long solids retention times provided by a batch-loaded aerobic fluidized-bed reactor were required to successfully treat stormwater containing ethylene glycol (Saffermann et al., 1998). Nitschke et al. (1996) found that activated sludge had to be adapted to ADF waste for several days to meet biochemical oxygen demand (BOD) and chemical oxygen demand (COD) discharge limits. The negative effects on wastewater treatment facilities caused by shock loadings of ADF wastes have caused some utilities to begin enforcing strict discharge limits and assessing substantial fees to treat ADF-containing waste streams from airports. As a result, airports and airbases are investigating alternative treatment methods for ADF wastes.

Because of the extremely high oxygen demand associated with the glycol content of deicing fluids, ADF-containing waste streams have been considered for anaerobic treatment in lagoons and high-rate digestion systems. The primary constituent of ADF waste, propylene glycol (PG), has been shown to be easily degraded under anaerobic conditions (Veltman et al., 1998). At Albany County Airport (Albany, New York), an anaerobic fluidized-bed reactor (AFBR) with activated carbon as the support medium has been dedicated for the treatment of ADF waste. During pilot testing, 92 to 99% COD removal with a loading rate as great as 15 kg COD/m^3·d was achieved (AST et al., 1998).

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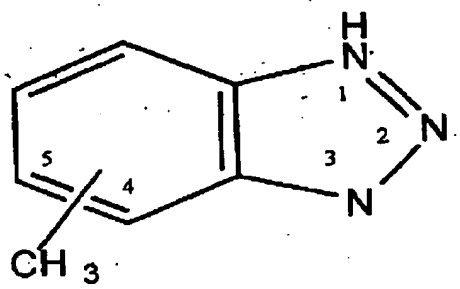

Figure 1—4-(5-) Methylbenzotriazole.

There are no data available, however, on the fate and treatability of the special-purpose chemical additives present in modern ADF or their effects on the biodegradation rates of the glycols that compose most of the ADF.

Some wastewater that shows good potential for anaerobic treatment, like ADF waste, may contain levels of certain chemicals that are toxic to anaerobic bacteria or are otherwise inhibitory to the anaerobic digestion process. A typical mechanism for anaerobic process toxicity is the adverse effect of a substance on methanogenic activity. Methanogenic bacteria, in particular, are considered sensitive microorganisms in the consortium of obligate anaerobic bacteria grown in anaerobic digesters and are often the phylum most adversely affected by a toxicant (McCarty, 1964). Classical anaerobic toxicity assays survey indirect indicators of methanogenic toxicity such as accumulation of volatile fatty acids (VFAs), reduction in methane production, or decreased substrate removal efficiency (Lo and Hegemann, 1998; Owen et al., 1979; and Uberoi and Bhattacharya, 1997). The limitations of traditional toxicity assays prohibit rigorous investigations to the response of the microbial community in anaerobic treatment systems when challenged with inhibitory waste streams. An understanding of microbial community structure and its effect on treatment efficiency is essential for the development of successful treatment processes (Raskin et al., 1994a). Indirect indicators of anaerobic toxicity (chemical markers) may be unstable and may not provide information on the viability of the specific microbial communities responsible for carrying out anaerobic treatment.

The application of comparative ribosomal RNA (rRNA) sequencing eliminates problems of culturability and permits the direct study of microbial ecology in natural and engineered systems (Christensen and Poulsen, 1994; Pace et al., 1986; and Wagner et al., 1994). Quantification of the activity of bacterial populations can be estimated from hybridization of labeled oligonucleotide probes to rRNA. The cellular ribosome content and, consequently, the rRNA concentration vary with growth rate. Toxicity to specific populations can be identified directly by a noted reduction in number of cells or microbial activity (as judged by RNA content). Single cells can be identified and quantified based on whole-cell hybridization of fluorescent-dye oligonucleotide probes to rRNA (Amman et al., 1990; Raskin et al., 1994a; and Spear et al., 1999). Whole-cell hybridization allows individual members of consortia to be identified and enumerated intact. Therefore, changes in community structure caused by environmental conditions and inhibitory substances can be monitored.

Research Objectives. This research had three objectives: to investigate the effects of the corrosion inhibitor (MeBT) added to ADF on anaerobic microbial communities actively co-digesting wastewater sludge and the main ingredient of ADF, PG; compare the toxic response of MeBT as judged by conventional anaerobic toxicity assays to the response indicated by quantitative fluorescent in situ hybridization (FISH) with phylogenetic probes; and determine the fate of MeBT through anaerobic digestion.

Materials and Methods

Bench-Scale Anaerobic Digesters. Two bench-scale anaerobic digesters were established in 4-L Erlenmeyer flasks. The digesters used in this research were seeded with actively digesting sludge collected from the 6.4×10^4 m³/d Boulder Wastewater Treatment Facility (BWWTP) in Boulder, Colorado, which is a trickling filter–solids contact facility with mesophilic anaerobic digesters. Laboratory digesters were stirred intermittently with Teflon-coated stir bars (1.5 cm diam. × 20 cm length) and shaken vigorously every 48 hours to ensure adequate mixing. The digesters were operated at 37 ± 2 °C. These bench-scale digesters were maintained with a feed of sludge from primary sedimentation tanks (at BWWTP) amended with PG (80% of total COD). An occasional pH adjustment was necessary to stabilize the bench-scale digesters when acclimating them to PG; pH was maintained at 7.0 ± 0.2 units through addition of sodium bicarbonate (75 g/L). These digesters were operated at a 15-day solids retention time (SRT) for a period of 6 months before challenging their PG-acclimated biomass with MeBT. The PG-acclimated biomass was used for both sorption assay and anaerobic toxicity assays of MeBT. Operating parameters of these bench-scale digesters are listed in Table 1.

Sorption Studies. Batch experiments were performed to characterize the sorptive behavior of MeBT in anaerobic digesters. Under a headspace of 20% CO_2 balanced with H_2, 10 mL of digesting sludge was pipetted into a 40-mL scintillation vial containing ultrapure water (Milli-Q, Millipore Corporation, Bedford, Massachusetts) and MeBT. Headspace was eliminated, and vials were sealed with screw caps containing Teflon-coated inserts. The minimum time required for complete sorption was determined in preliminary experiments. After 36 hours of exposure to digesting sludge, soluble MeBT concentration remained unchanged. The vials with varying concentrations of MeBT (100 to 1000 mg/L) were allowed to equilibrate on a shaker table at 37 ± 2 °C for approximately 48 hours to ensure complete sorption. Each sample was prepared in triplicate.

Table 1—Digester operating parameters.[a]

Parameter	Units	Range	Mean
Loading Rate	mg COD/L·d	6950–7500	7350
pH		6.9–7.2	7.0
Alkalinity	mg $CaCO_3$/L	1500–2800	2000
Methane	%	60–79	71
Carbon dioxide	%	27–37	31
Temperature	°C	36–39	36
Volatile fatty acids (C_2–C_5)	mg/L	180–225	200
Volatile solids	g/L	2.5–3.7	3

[a] Solids retention time = 15 days.

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Anaerobic Toxicity Assays. The methods reported by Donlon et al. (1995) were modified according to the following procedures. Toxicity assays were carried out in 125-mL serum vials (Wheaton No. 223748, Millville, New Jersey) with gray butyl rubber stoppers and 20-mm aluminum crimp seals. Digesting sludge (75 mL) was pipetted into serum vials that served as easily replicated digester microcosms. Methylbenzotriazole (0 to 1000 mg/L final concentration) was added to each microcosm. A mixture of primary sludge centrate and PG was used as substrate (total volume = 6 mL, COD added = 17 000 mg/L). Each serum vial was topped off with Milli-Q leaving a 25-mL headspace. The headspace was flushed with 20% CO_2 balanced with H_2 before incubation. Each sample was prepared in triplicate. The microcosms were allowed to digest for 48 hours on a shaker table at 37 ± 2 °C.

After the initial 48-hour incubation, the serum vials were fed again, and methane monitoring began. The headspace of the serum vial was flushed daily with 20% CO_2 balanced with H_2 for accurate monitoring of methane production. Headspace methane production was analyzed following this second feeding at 3, 6, 9, 12, 16, 24, and 36 hours, and then daily for 5 days to observe the response to the toxicant (MeBT). Total solids (TS), volatile solids (VS), COD, and VFA analyses were performed at the beginning and end of the incubation period. The serum vial microcosm experiment was repeated with the addition of granular activated carbon (GAC) to the cultures (VS:GAC = 10%) for 0, 500, and 1000 mg/L MeBT. A one-tailed, paired *t*-test ($\alpha = 0.05$, whose α is the probability that the two averages are not statistically different) was used to determine whether there were significant differences among the cumulative methane, VS, and VFAs produced in microcosms containing varying concentrations of MeBT, GAC, and those serving as controls (no MeBT).

Methane. Headspace biogas concentrations were analyzed using a gas chromatograph with a thermal conductivity detector (TCD) (Gow-Mac Instrument Co., Bethlehem, Pennsylvania). The GC was fitted with a 3 mm × 2.4 m Haysep Q capillary column (Supelco, Inc., Bellefonte, Pennsylvania), and column temperature was maintained at 80 °C. The flow rate of the carrier gas, helium, was 20 mL/min. The TCD temperature was maintained at 100 °C. A personal computer was used to record and measure the data generated (Peak Simple, SRI Instruments, Torrance, California). Injections were made with a 250-µL gastight syringe (Hamilton, Inc., Reno, Nevada).

Methylbenzotriazole. Analysis for MeBT concentration in digesting sludge was performed using a Shimadzu (Columbia, Maryland) high-pressure liquid chromatograph fitted with a UV detector (at a wavelength of γ_{254}). Samples were centrifuged and the centrate was filtered (0.2 µm) before analysis. MeBT separation was achieved isocratically using an Inertsil ODS-3 250 x 4.6 mm column (MetaChem Technologies, Inc., Torrance, California) and an eluant composed of 50% methanol, 49% Milli-Q, and 1% acetic acid (v/v) at a flow rate of 1.5 mL/min and an injection volume of 20 µL. The method detection limit was 0.5 mg/L MeBT.

Volatile Fatty Acids. Volatile fatty acids were measured using a gas chromatograph (HP5890, Hewlett Packard, Corvallis, Oregon) fitted with an SPB-1000 capillary column (Supelco, Inc.). Nitrogen was used as the carrier gas (20 mL/min). Column, injector, and detector temperatures were 185, 220, and 250 °C, respectively. Samples were centrifuged, and the centrate was filtered (0.45 µm) and acidified (pH < 2) with sulfuric acid before analysis.

Fluorescent In Situ Hybridization. Two oligonucleotide probes, circumscribing the domains Archaea and Bacteria, were used for FISH analysis (Amann et al., 1995) on anaerobic digesting sludge samples (Table 2). These oligonucleotides (Genosys Biotechnologies, Inc., Woodlands, Texas) have been well characterized and used successfully in many environments, including anaerobic digesters (Amann et al., 1990; Merkel et al., 1999; Raskin et al., 1994a and 1994b; and Stahl and Amann, 1991).

Table 2—Oligonucleotide probes.

Probe		Td, °C
ARC915: Archaea (Stahl and Amann, 1991) Label: CY3	5'-GTGCTCCCCCGCCAATTCCT Target Site: (915–924)	58
EUB338: Bacteria (Amann et al., 1990) Label: Oregon Green	5'-GCTGCCTCCCGTAGGAGT Target Site: (338–355)	52

Cell Fixation and Whole-Cell Hybridization. Cell fixation and hybridization methods were modified from those previously reported by Raskin et al. (1994a and 1994b). The fixative was prepared immediately before use. Approximately 2 mL of sample were withdrawn from microcosms using a 5-mL syringe fitted with a 20 gauge needle and transferred to a 1.7-mL microcentrifuge tube. Samples were sonicated for 10 minutes then centrifuged at 500 r/min for 5 minutes. One milliliter of the centrate was transferred to a new microcentrifuge tube and centrifuged at 7000 r/min for 2 minutes to form a pellet. The centrate was poured off and cells were resuspended in fixative. The fixed cells were stored at −20 °C for up to 7 days before microscope enumeration. Fixed cells were added to hybridization solution with approximately 4 µL of fluorescently labeled oligonucleotide probe at 0.5 µg/µL and incubated at 37 ± 2 °C for 12 to 18 hours. Cells were then placed in an oven for 30 minutes for washing at the published hybridization temperature (Table 2).

Filtration and Enumeration. Hybridized cells were filtered through a 0.2-µm black polycarbonate filter (Poretics, Cat. No. K02BP02500, Oakley, California) backed with a 5-µm silver membrane (Osmonics, Cat. No. 46680, Oakley, California). To facilitate a uniform distribution of bacteria on filters, approximately 25 mL of filtered (0.2 µm) Milli-Q was placed in the filtration funnel before addition of the hybridized cells. Between 100 and 250 µL of hybridized cells were pipetted into the filter funnel, and 4′,6-diamidino-2-phenylindole (DAPI) was added to a final concentration of 5mM. The solution was carefully mixed with a pipettor. After 5 minutes of contact with DAPI, the samples were filtered and allowed to dry before placement on a frosted slide. A drop of immersion oil was dispensed between the slide and the coverslip, and slides were stored in the dark before viewing under the microscope. One slide was prepared for each of the triplicate batch assays. A minimum of seven fields and 350 of each fluorescently labeled microorganism (ARC915, EUB338, and DAPI) were counted per slide. The ratio of the number of the cells in domains Archaea and Bacteria to total DAPI counts were used for comparing sample cultures.

Results

Batch Sorption Studies. A Freundlich isotherm described the sorptive behavior of MeBT on anaerobic digesting sludge in the

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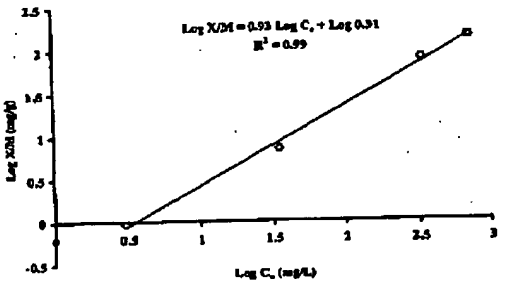

Figure 2—Sorption of MeBT to digester sludge. Error bars represent 99% confidence intervals; *R* = regression coefficient; *X/M* = mg of sorbed MeBT/g VS; and C_e = aqueous concentration of MeBT.

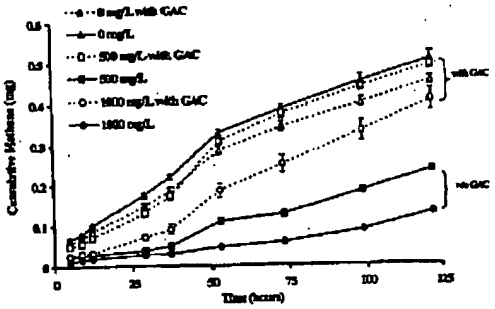

Figure 4—Methane production in anaerobic digester microcosms with different MeBT concentrations in the presence and absence of GAC (VS:GAC = 10%). Error bars represent ± 1 standard error.

concentration range observed. Between 10 and 30% of the MeBT (100 to 1000 mg/L) introduced to the systems sorbed to the digesting sludge, which was on average 1.5% TS. Both 4- and 5-MeBT isomers sorbed to anaerobic digester sludge; a Freundlich isotherm model described this sorption. Experimental results are summarized in Figure 2.

Methylbenzotriazole Stability. Both 4- and 5-MeBT persisted in all microcosms and bench-scale digesters. No breakdown of MeBT was detected during any of the anaerobic toxicity assays or in bench-scale anaerobic digesters maintained to observe the behavior of MeBT over an 18-month period.

Anaerobic Toxicity Assays. The MeBT was added to anaerobic microcosms in increasing concentrations (100 to 1000 mg/L) to assess anaerobic toxicity. Cumulative methane production was measured for a period of 5 days following staged feedings of digester microcosms. Methane production decreased in response to increases in MeBT concentration. Compared to digesters fed only settled domestic wastewater and PG, MeBT concentrations of 300 mg/L and greater caused a statistically significant decrease in methanogenic microbial activity after approximately 50 hours of incubation (one tailed *t*-test, $\alpha = 0.05$) (Figure 3). In microcosms containing approximately 1000 mg/L MeBT, methane production was less than 50% of the control. As shown in Figure 4, addition of GAC (VS:GAC = 10%) to microcosms containing identical concentrations of MeBT resulted in a statistically significant increase in methane production (one tailed *t*-test, $\alpha = 0.05$) compared to control systems without GAC. Methane production from microcosms containing 500 mg/L MeBT in the presence of GAC increased 250%. However, the methane production in the presence of GAC (no MeBT) was significantly lower than the control (no GAC and no MeBT). Error bars represent ± 1 standard error around the mean of triplicate measurements except where otherwise noted. In some instances, error bars were smaller than the symbols.

Volatile Solids Production. The change in VS (i.e., observed yield) typically decreased over the duration of the microcosm experiments in response to increases in MeBT concentration (Figure 5). At 100 mg/L MeBT or greater, a statistically significant decrease in VS was observed (one-tailed *t*-test, $\alpha = 0.05$). There

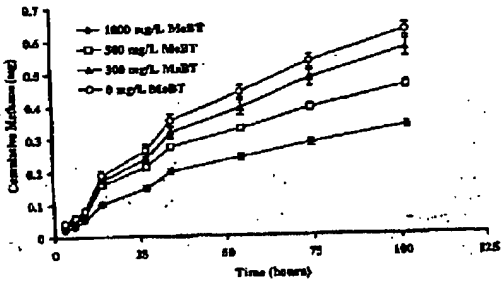

Figure 3—Methane production in anaerobic digester microcosms with different MeBT concentrations. Error bars represent ± 1 standard error.

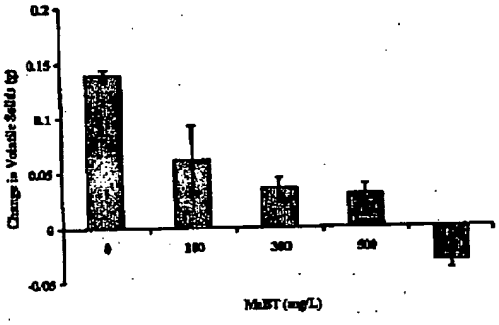

Figure 5—Change in VS concentrations during anaerobic toxicity assays. Error bars represent ± 1 standard error.

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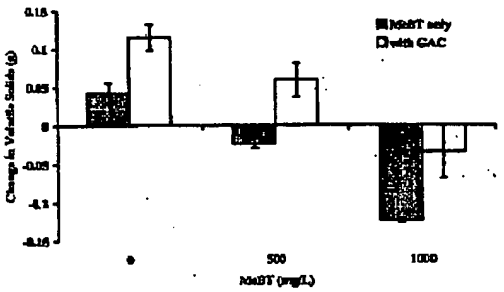

Figure 6—Change in VS concentrations during anaerobic toxicity assays in the presence and absence of GAC (VS:GAC = 10%). Error bars represent ± 1 standard error.

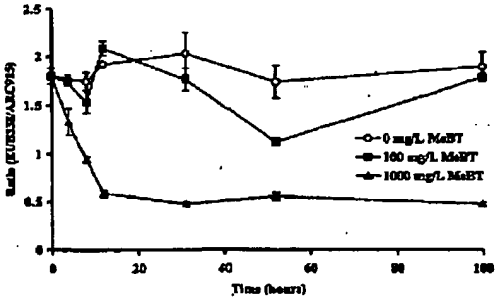

Figure 8—Relative response of Archaea and Bacteria to increasing concentrations of MeBT during anaerobic toxicity assays.

was a net loss of VS when MeBT concentrations were 1000 mg/L. Compared to control systems, GAC had a significant effect on VS production when MeBT was present (Figure 6). In the absence of GAC, there was a decline in VS over the duration of the anaerobic toxicity assay at 500 and 1000 mg/L MeBT; however, the addition of GAC in the presence of 500 mg/L MeBT resulted in an increase in VS production during the period of observation. The VS increase of the controls (no MeBT) was significantly greater in the presence of GAC.

Volatile Fatty Acid Formation. Figure 7 summarizes the VFA (C_2 to C_5) concentrations in microcosms with varying concentrations of MeBT (100 to 1000 mg/L). Total VFA concentration was calculated by summing all of the measured VFAs. Valeric, butyric, and isobutyric acids were only measurable in microcosms containing MeBT. Propionic acid is an intermediate in the anaerobic biodegradation of PG (Veltman et al., 1998). Propionic acid was formed in response to MeBT and composed most of the total VFAs for all samples containing MeBT. Propionic and total VFA concentrations increased in response to increasing MeBT levels up to 500 mg/L; however, at 1000 mg/L MeBT, propionic acid and total VFA concentrations showed a sharp, statistically significant decrease. Total VFA and propionic acid concentrations were significantly reduced in the presence of GAC. In all cases, standard errors of VFA analyses were less than 10% of the calculated VFA concentration.

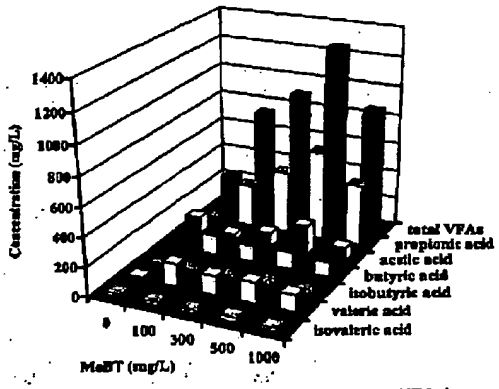

Figure 7—Effect of MeBT concentration on VFA formation during anaerobic toxicity assays.

Fluorescent In Situ Hybridization. Figure 8 presents the effect of increasing MeBT concentration on the numbers of active cells in the domains Archaea and Bacteria during a 5-day incubation period. The number of active cells in the domain Bacteria was approximately twice the number of active cells in the domain Archaea when no MeBT was present or below the toxicity threshold (< 300 mg/L). At 1000 mg/L MeBT, the EUB338:ARC915 ratio decreased significantly (from 2 to 0.5) within 12 hours and remained at approximately 0.5 for the subsequent 4 days. At a high concentration (1000 mg/L MeBT), the members of the domain Bacteria present in the digesters were more sensitive to MeBT than were the members of the domain Archaea.

Direct microscopic measurements of fluorescent phylogenetic probes applied to digesting biomass revealed that members of both the domains Archaea and Bacteria present in the digester microcosms were sensitive to MeBT (Figure 9). The percent of active Bacteria (EUB338:DAPI) and the percent of total active cells ((EUB338+ARC915):DAPI) decreased in response to increasing MeBT concentration. As judged by DAPI, the number of total DNA-containing cells dropped significantly in response to MeBT (MeBT > 500 mg/L). The percent of active Archaea (ARC915:DAPI) increased slightly at 500 and 1000 mg/L MeBT; this was the result of concurrent but greater loss of active members of the domain Bacteria and loss of DNA-containing cells (DAPI) in response to greater MeBT concentrations. In all cases, the standard error of the ratio was less than 11% using standard propagation of random error calculations (Miller and Miller, 1988).

Discussion

In batch studies, the log-linear sorption of MeBT to anaerobic digesting sludge was significant at 1.5% TS. The Freundlich constants were $1/n$ (slope) = 0.93 and K_f (adsorption coefficient) = 0.31. These results suggest that, depending on solids retention time

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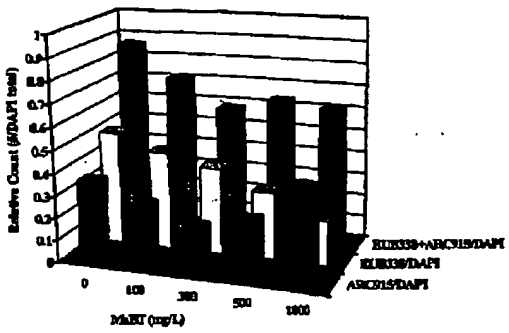

Figure 8—Archaea and Bacteria 48-hour response to increasing concentrations of MeBT during anaerobic toxicity assays.

and loading rates, accumulation of MeBT may occur in anaerobic digesters because of its sorptive interaction with digesting solids. In the presence of GAC (VS:GAC = 10%), no MeBT was found in the liquid phase (0.45-μm filtered) under any conditions, suggesting that MeBT may be sequestered out of the liquid phase by GAC.

The MeBT inhibited the methanogenic activity of otherwise healthy anaerobic biomass co-digesting PG and settled wastewater. A toxicity threshold, defined as the concentration where a statistically significant decrease in methane production was measured, was observed at 300 mg/L MeBT. As MeBT concentrations increased above this threshold, methane production significantly decreased; high concentrations of MeBT (1000 mg/L) were inhibitory to methanogenic activity, reducing it by more than 50% (Figure 3).

Many deicing operations discharge their waste directly to local wastewater treatment facilities through sanitary sewer systems. Some airports and military airbases are instituting on-site treatment alternatives for ADF-containing wastes (e.g., Albany County Airport) and most are attempting to collect ADF waste at the point of application, resulting in more concentrated waste streams. Deicing operations typically produce ADF wastes with strengths ranging between 1 and 15% of the original PG concentration (15% is the level that makes PG recycling economically viable). Anaerobic treatment systems accepting flows from deicing operations may receive ADF waste containing 6% PG and approximately 300 mg/L MeBT, which was the toxicity threshold observed in these experimental systems. This corresponds to market-grade deicing fluids diluted between 6 and 60 times, with inhibitory effects becoming apparent at dilutions of approximately 20 times.

Granular activated carbon addition significantly reduced the adverse effects of MeBT on mesophilic digestion. Volatile solids production (Figure 6), VFA accumulation, and methane production (Figure 4) were all maintained near levels observed in the control systems when GAC was present. Anaerobic systems can treat wastewater that contains a toxicant if the toxicant is sequestered or chelated (i.e., not biologically available) under treatment conditions. In these experiments, the activated carbon added to the microcosms adsorbed the MeBT and apparently reduced its availability to digesting biomass, mitigating the negative effects of MeBT on the digestion processes. Anaerobic fluidized-bed reactors using GAC media have been reported to successfully treat coal gasification waters containing relatively toxic nitrogen-conjugated aromatic compounds (Berchtold et al., 1995, and Wang et al., 1984). Highly inhibitory wastewater containing chlorophenols and 2,4-dinitrotoluene has also been treated effectively using anaerobic fluidized-bed reactors (Berchtold et al., 1995, and Suidan et al., 1996). In this research, the addition of GAC effectively increased the toxicity threshold of biomass digesting a waste stream containing MeBT by decreasing its aqueous concentration. The differences in methane production observed between control microcosms with and without GAC may be attributed to decreased substrate availability caused by sorption (Figure 4). In addition, the increased VS observed in the controls with the addition of GAC may be the result of the development of additional biomass attached to the GAC or the sequestering of other potentially toxic substrates in the feed solution (Figure 6).

Three different stains were used to determine the relative response of digesting biomass to increasing MeBT concentrations; two phylogenetic probes were used to independently track the activity of members of the domains Archaea and Bacteria, and DAPI, a nonspecific DNA intercalating agent, was used to determine the total numbers of cells present regardless of their activity. The two domain-level probes were chosen because they circumscribe the largest collective domains of bacteria present in anaerobic digesters, which in turn correspond with two primary classes of microbiological activity that occur during anaerobic digestion: acidogenesis and methanogenesis. Conventionally, the activity of acidogenic and methanogenic populations in anaerobic digesters has been monitored indirectly using VFA and methane measurements collectively. The probe ARC915 was chosen in this work because methanogens are the only Archaea that are not found in extreme environments (e.g., thermophiles). Although the true ecology of acidogenic bacteria has not been comprehensively described, the domain-level EUB338 probe was used to measure the activity of other nonmethanogenic bacteria present in the digesters, including acidogenic populations (Merkel et al., 1999).

The MeBT had a significant effect on the digesting biomass as judged by the recovery of these sensitive biological stains. The total number of cells containing DNA (DAPI counts) decreased by approximately 15 ± 2% in the range of MeBT concentrations greater than 500 mg/L. This suggests that MeBT caused the lysis of cells at more than this concentration or that MeBT changed the conformation of intracellular DNA. At a high concentration of MeBT (1000 mg/L), the microbial ecology in the anaerobic toxicity assay changed; Bacteria became the dominant population (EUB:ARC = 2.0) (Figure 8). Through the range of concentrations observed (100 to 1000 mg/L MeBT), phylogenetic probes concurred with traditional toxicity assays, indicating that members of the domain Archaea were sensitive to MeBT (Figure 9). At MeBT concentrations less than 500 mg/L, MeBT had a greater effect on members of the domain Archaea than members of the domain Bacteria; this is supported by the direct counts of probe-hybridized bacteria (normalized to DAPI) and increased VFA levels observed in this concentration range.

Analyzing the data from the anaerobic toxicity assays (such as methane production) did not give complete information on the effect of MeBT on the ecological response of the mesophilic anaerobic digesters used in this study. The decrease in methane production indicated sensitivity of members of the domain Archaea to MeBT at all concentrations studied. Measurements of

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fluorescent phylogenetic probes (Figure 9) and VFA concentrations in response to increasing MeBT levels suggest that members of the domain Bacteria present in the digesters were sensitive to MeBT at all concentrations studied, and that they were as sensitive or more sensitive to greater concentrations (> 500 mg/L) of MeBT than were members of the domain Archaea. At 1000 mg/L MeBT, VFA production was significantly lower than at 500 mg/L. Volatile solids and methane production response were also low in this concentration range. The VFA and probe data confirm that both acidogenic and methanogenic populations were inhibited at concentrations greater than 500 mg/L (Lu and Hegemann, 1998).

Conclusions

Significant amounts of the MeBT fed to anaerobic digesters sorbed to digesting sludge. Between 10 and 30% of the MeBT (100 to 1000 mg/L) introduced to the system sorbed to the digester sludge (1.5% TS). Both 4- and 5-MeBT sorbed to anaerobically digesting sludge; this sorption was well approximated by a Freundlich isotherm model according to the following empirical relation: $\log X/M = 0.93 \log C_e + \log 0.31$. No breakdown of MeBT was detected during any of the anaerobic toxicity assays or in bench-scale anaerobic digesters maintained to observe the behavior of MeBT over an 18-month period. These observations suggest that MeBT is recalcitrant to anaerobic degradation and will likely be present in digester supernatant. Digesters fed MeBT concentrations greater than 300 mg/L responded with a significant decrease in methanogenic microbial activity and VS production and a concomitant increase of VFA concentrations. Direct microscopic measurements of fluorescent phylogenetic probes applied to digesting biomass revealed that (a) at concentrations of 100 mg/L and greater, members of the domains Archaea and Bacteria were sensitive to the presence of MeBT and (b) members of the domain Bacteria present in the digesters were as sensitive or more sensitive to MeBT than were members of the domain Archaea at concentrations greater than 500 mg/L. The results observed for population activity measured by FISH corresponded to those observed for the anaerobic toxicity assays; however, the anaerobic toxicity assay could not resolve concurrent toxic responses of cells from both domains (Archaea and Bacteria). The addition of GAC to an anaerobic treatment system for ADF waste may diminish the toxic effects of MeBT and eliminate MeBT from the effluent.

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Authors. Cyndee Gruden is an adjunct professor at Colorado School of Mines, Golden, Colorado. Sarahann Dow is a graduate research assistant and Mark Hernandez is an associate professor in the Department of Civil, Environmental, and Architectural Engineering at the University of Colorado at Boulder. Correspondence should be addressed to Mark Hernandez, Department of Civil, Environmental, and Architectural Engineering, Campus Box 428, University of Colorado, Boulder, CO 80309-0428.

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References

Amann, R.I.; Binder, B.J.; Olsen, R.I.; Chisholm, S.W.; Devereux, R.; and Stahl, D.A. (1990) Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations. *Appl. Environ. Microbiol.*, 56, 1919.

Amann, R.I.; Ludwig, W.; and Schleifer, K.-H. (1995) Phylogenetic Identification and In-Situ Detection of Individual Cells without Cultivation. *Microbiol. Rev.*, 59, 143.

Applied Science and Technology; Rensselaer Polytechnical Institute; and University of Massachusetts, Amherst (1998) Anaerobic Treatment of Deicing Waste—A Technology Assessment. New York State Energy Research and Development Authority, Albany, N.Y.

Berchtold, S.R.; VanderLoop, S.L.; Sufdan, M.T.; and Maloney, S.W. (1995) Treatment of 2,4-Dinitrotoluene Using a Two-Stage System: Fluidized Bed Anaerobic GAC Reactors and Aerobic Activated Sludge Reactors. *Water Environ. Res.*, 67, 1081.

Cancilla, D.A.; Holtkamp, A.; Matassa, L.; and Fang, X. (1997) Isolation and Characterization of Microtox-Active Components from Aircraft De-Icing/Anti-Icing Fluids. *Environ. Toxicol. Chem.*, 16, 430.

Cancilla, D.A.; Martinez, J.; and Aggelen, G.C.V. (1998) Detection of Aircraft Deicing/Antiicing Fluid Additives in a Perched Water Monitoring Well at an International Airport. *Environ. Sci. Technol.*, 32, 3834.

Christensen, H., and Poulsen, L.K. (1994) Detection of *Pseudomonas* in Soil By rRNA Targeted In Situ Hybridization. *Soil Biol. Biochem.*, 26, 1093.

Cornell, J.S.; Pillard, D.A.; and Hernandez, M. (2000) Comparative Measures of the Toxicity of Component Chemicals in Aircraft Deicing Fluids. *Environ. Toxicol. Chem.*, 19, 6.

Donlon, B.A.; Razo-Flores, E.; Field, J.A.; and Lettinga, G. (1995) Toxicity of N-Substituted Aromatics to Acetoclastic Methanogenic Activity in Granular Sludge. *Appl. Environ. Microbiol.*, 61, 3889.

Downs, W.R. (1968) Chemically Induced Ignition in Aircraft and Spacecraft Electrical Circuitry by Glycol/Water Solutions. Tech. Rep., NASA Manned Spacecraft Center Houston, Washington, D.C.

Krumsick, T.A.; Dnigger, G.T.; and Farmer, W. (1994) Glycol Wastewater Treatability Study. *Proc. 67th Annu. Water Environ. Fed. Conf. Exposition*, Chicago, Ill.

Lu, Z., and Hegemann, W. (1998) Anaerobic Toxicity and Biodegradation of Formaldehyde in Batch Cultures. *Water. Res.* (G.B.), 32, 209.

McCarty, P.L. (1964) Anaerobic Waste Treatment Fundamentals—Part Three—Toxic Materials and Their Control. *Pub. Works*, 11, 91.

Merkel, W.; Manz, W.; Szewzyk, U.; and Krauth, K. (1999) Population Dynamics in Anaerobic Wastewater Reactors: Modeling and In Situ Characterization. *Water Res.* (G.B.), 33, 2392.

Miller, J.C., and Miller, J.N. (1988) *Statistics for Analytical Chemistry*. 2nd Ed., Wiley & Sons, N.Y.

Nitschke, L.; Wagner, H.; Metzner, G.; Wilk, A.; and Huber, L. (1996) Biological Treatment of Waste Water Containing Glycols from Deicing Agents. *Water Res.* (G.B.), 30, 644.

Owen, W.F.; Stuckey, D.C.; Healy J.B.; Young, L.Y.; and McCarty, P.L. (1979) Bioassay for Monitoring Biochemical Methane Potential and Anaerobic Toxicity. *Water Res.* (G.B.), 13, 485.

Pace, N.R.; Stahl, D.A.; Lane, D.J.; and Olsen, G.J. (1986) The Analysis of Natural Microbial Populations by Ribosomal RNA Sequences. *Adv. Microbiol. Ecol.*, 9, 1.

PMC Specialties Group, Incorporated (1996) COBRATEC® TT-100 Technical Bulletin. Tech. Rep., Rocky River, Ohio.

Rao, N.M.; Lu, F.F.; Johnson, D.A.; and Nghiem, N.P. (1996) Method of Preventing Yellow Metal Corrosion in Aqueous Systems with Superior Corrosion Performance and Reduced Environmental Impact. U.S. Patent 5,503,775, Nalco Chemical Company.

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Raskin, L.; Poulsen, L.K.; Noguera, D.R.; Rittmann, B.E.; and Stahl, D.A. (1994a) Quantification of Methanogenic Groups in Anaerobic Biological Reactors by Oligonucleotide Probe Hybridization. *Appl. Environ. Microbiol.*, 60, 1241.

Raskin, L., Stromley, J.M.; Rittmann, B.E.; and Stahl, D.A. (1994b) Group-Specific 16S rRNA Hybridization Probes to Describe Natural Communities of Methanogens. *Appl. Environ. Microbiol.*, 60, 1232.

Saheb, Y., and Narasiah, K.S. (1992) Degradation Rate of Aircraft Deicing Fluid in a Sequential Biological Reactor. *Water Sci. Technol.*, 26, 2061.

Seffernan, S.L.; Shruvalmr, G.S.; and Poppe, L.B. (1998) Deicing Fluid Treatment in Batch-Loaded Aerobic Fluidized Bed Reactor. *J. Environ. Eng.*, 124, 11.

Society of Automotive Engineers (1997) Specification 1424c, Deicing/Anti-icing Fluid, Aircraft, SAE Type I. Soc. Auto. Eng. Aerospace Matrr. Specific., Washington, D.C.

Spear, R.N.; Li, S.; Nordheim, E.V.; and Andrews, J.H. (1999) Quantitative Imaging and Statistical Analysis of Fluorescence in Situ Hybridization (FISH) of *Aureobasidium pullulans*. *J. Microbiol. Meth.*, 35, 101.

Stahl, D.A., and Amann, R. (1991) Development and Application of Nucleic Acid Probes. *Nucleic Acid Techniques in Bacterial Systematics*. Wiley & Sons, N.Y., 205.

Suidan, M.T.; Flora, J.R.; Boyer, T.K.; Wuellner, A.M.; and Narayanan, B. (1996) Anaerobic Dechlorination Using A Fluidized-Bed GAC Reactor. *Water Res.* (G.B.), 30, 1, 160.

Uberoi, V., and Bhattacharya, S.K. (1997) Toxicity and Degradability of Nitrophenols in Anaerobic Systems. *Water Environ. Res.*, 69, 146.

U.S. Environmental Protection Agency (1999) Airport Deicing Operations Study. Draft Effluent Guidelines. EPA-821/d-99-002, Off. Water, Washington, D.C.

Veltman, S.; Schoenberg, T.; and Switzenbaum, M.S. (1998) Acid and Alcohol Formation During Propylene Glycol Degradation under Anaerobic Methanogenic Conditions. *Biodegradation*, 9, 113

Wagner, M.; Erhart, R.; Manz, W.; Amann, R.; Lemmer, H.; Wedi, D.; and Schleifer, K.H. (1994) Development of an rRNA-Targeted Oligonucleotide Probe Specific for the Genus *Acinetobacter* and Its Application for In Situ Monitoring of Activated Sludge. *Appl. Environ. Microbiol.*, 60, 792.

Wang, Y.; Suidan, M.T.; and Pfeffer, J.T. (1984) Anaerobic Activated Carbon Filter for the Degradation of Polycyclic N-Aromatic Compounds. *J. Water Pollut. Control Fed.*, 56, 1247.